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Forensic blood testing after long-term exposure to chemical interferents

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FORENSIC BLOOD TESTING AFTER LONG-TERM EXPOSURE TO CHEMICAL INTERFERENTS

by

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ABSTRACT

The field of forensic science encounters many types of evidence. To narrow down what is probative, there are presumptive and confirmatory tests that may be performed on scene and in the laboratory. Most of these tests are performed with one goal in mind: to provide linkages. Biological linkages are made via deoxyribonucleic acid (DNA) left behind on evidence. Blood is the most common source of DNA for investigators. Blood analysis is performed in a specific order, starting with a visual inspection then presumptive testing, and lastly confirmatory testing. Typically, if an item yields a negative presumptive test then confirmatory testing isn't necessary. However, some tests that yield a negative presumptive result aren't necessarily indicative of the absence of blood. Interferents contained in common household cleaning products and other items can potentially cause false negative results.

This research was a continuation of an earlier study from approximately three years ago that explored the relationship between interferents and false negative results during blood analysis. That study concluded that stronger chemical interferents resulted in more false negatives. It also found that less concentrated blood samples produced more frequent false negatives. A review of the literature reveals that several chemical interferents present in everyday household products have the ability to mask the presence of blood. A variety

of these common products were tested, including concentrated chemical interferents contained in these products.

Previously analyzed samples from 2021 were retested approximately three years later in this study for a results comparison. Specifically, this current study focused on the distribution of false negatives for both presumptive (Kastle-Meyer and Orthotolidine) and confirmatory (RSID™ and HemaTrace®) tests for blood. Detailed preliminary visual inspections were also performed. Upon inspection, many samples contained less than a 1:10 concentration of blood which was difficult to discriminate by eye. Typically, in forensic case work, a negative visual inspection for a stain would preclude further testing. In this study, false negatives results were obtained for most interferents during presumptive testing, while confirmatory tests yielded mostly positive results. Certain household cleaners yielded false negatives for both presumptive and confirmatory testing. In these instances, the apparent lack of blood evidence would likely preclude DNA testing.

Research that identifies factors that contribute to false negative blood test results is important for the forensic community. More sensitive and specific tests can help limit the discarding of potential forensic evidence. This experiment seeks to provide results that can be useful in casework, especially for potential blood evidence that has been exposed to similar chemical interferents over a long period of time.

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LIST OF ABBREVIATIONS

ALS	Alternate Light Source
BKF	Bar Keeper's Friend
DNA	Deoxyribonucleic acid
IRB	Institutional Review Board
KM	Kastle-Meyer
μL	Microliter
mL	Milliliter
nm	Nanometer
O-TOL	Orthotolidine
RBCs	Red blood cells
RSID	Rapid Stain Identification
WBCs	White blood cells

1. INTRODUCTION

1.1 Blood Properties

Blood is one of the most common types of biological evidence found at a crime scene and various facets of its examination can provide investigators a great deal of information regarding victims and perpetrators. The properties of blood allow forensic scientists to test and collect it from crime scenes, while further in-depth laboratory testing can potentially link its genetic material to its donor using deoxyribonucleic acid (DNA).

Understanding the composition of blood is important when considering its testing and preservation in forensic testing. There are 4 primary components of blood: red blood cells (RBCs), white blood cells, (WBCs), platelets, and plasma. These components contribute to various biological functions and their unique attributes play a part in their forensic relevance such as providing genetic material for identification or blood typing.

WBCs, or leukocytes, make up around 1% of blood. WBCs are the components of blood that are responsible for immune response (1). Each of the 3 types of leukocytes (granulocytes, lymphocytes, monocytes) have a specific role in the immune system. They are distinguishable by microscopic properties and vary in quantity throughout blood. Of these 3 types, the lymphocytes branch out further into different types of cells based on their different functions. Neutrophils are part of the granulocyte branch and are the most abundant type of WBCs (50%-70% of WBCs) and are the first responders to fight an infection (2). DNA is contained in the WBC nucleus (3,4) and can provide forensic investigators important genetic information regarding its source.

Plasma is the liquid portion of blood and contains approximately 92% water. It also contains components that help blood clot, maintain pH, sustain osmotic pressure, and fight infection (5,6). Another component of blood is platelets. The function of platelets is vast, and they are responsible for many processes in the body. Platelets are important for inflammation, hemorrhages, clotting, injury healing, antimicrobial defense, tumor growth, and new blood cell formation (7). Both the plasma and red blood cells can be used for bloodstain analysis as well as estimating time of death.

RBCs, otherwise known as erythrocytes, are the most abundant cell in blood. They make up around 40-45% of the cells contained in whole blood (1). The main function of RBCs is to transport oxygen via the circulatory system from the lungs to the rest of the body using hemoglobin. Hemoglobin is a complex protein with 4 subunits, each containing a heme group. Heme is a ring-link molecule that contains an iron ion. Hemoglobin's unique structure is responsible for tightly binding the oxygen that gets distributed throughout the body (8). The heme component of blood is valuable for presumptive tests used in forensics. When heme is present, it catalyzes the color change of the presumptive tests. RBCs do not contain nuclei; therefore, they do not contain DNA.

1.2 Forensic Serology

Examining a crime scene or evidence using visual inspections and presumptive tests for the presence of blood is important in the recovery of potential DNA. However, there is an abundance of everyday items that can exhibit similar blood-like properties. A stain of red wine on a couch or a leftover piece of red meat on a kitchen knife can all mimic the appearance of blood and human tissue at a crime scene; hence, it is important to follow up with forensic confirmatory testing.

Typically, there is a specific sequence that is followed during the initial identification process for the presence of blood. A visual exam is completed first. This step includes visualization of the stain and its physical properties. Overall color, consistency, and texture are important features to note when deciding whether to continue with analysis. Once a reddish-brown stain is observed, the examiner moves forward with presumptive testing. Presumptive testing for blood is a useful tool for forensic scientists to help identify probative evidence. The options for presumptive blood tests are abundant, and their selection depends on the type of substrate, the conditions of the scene, and the product availability from the agency. Presumptive testing is then followed by more specific confirmatory testing.

1.2.1 Presumptive Testing for Blood

Catalytic color tests are widely used due to their sensitivity and ease of use. The reagents are cost effective, and the results are quick (15-30 seconds). An oxidation-reduction reaction causes the color change. When heme is present, it catalyzes a chemical reaction in which the colorless reagent become oxidized by hydrogen peroxide. This oxidization results in a characteristic color change.

Studies have shown sensitivity of catalytic color tests to be capable of detecting blood in a 1:100,000 dilution (9, 10). The quick results are also easy to interpret since a visible color change indicates a positive result. There are many reagents that can be used, and each comes with their own different colors, benefits and drawbacks. The more sensitive tests tend to be more carcinogenic due to the presence of benzene derivatives (11,12). Orthotolidine (O-TOL) may be more reliable in situations where there is a very small amount of blood present but is not as widely used because of the potential risks from the benzene (10). Also, the more sensitive tests are coupled with being less specific. Tests such as phenolphthalein, otherwise known as Kastle-Meyer (KM) are reportedly more specific than its counterparts, which can be a good choice for cases where blood and non-blood stains are potentially present (10).

Depending on lighting conditions, catalytic color tests that glow in darkness to highlight potential bloodstains may be utilized. These tests involve chemiluminescent reactions and are beneficial in locating latent bloodstains. Luminol, for example, utilizes the heme in blood to catalyze the oxidation of 3-aminophthalhydrazide found in the luminol reagent. The results develop in one step and a positive is indicated by light that is blue in color (13,14).

In addition to chemiluminescent reactions, fluorescence can also be used to locate blood presumptively. A chemical called fluorescein is used with an alternate light source (ALS) to display fluorescence when blood is present (15). Fluorescein reacts to the proteins and iron found in blood, specifically on the hemoglobin protein. The reaction occurs when these ions are exposed to light wavelengths between 420nm to 485nm which is in the blue light range (16,17). The glow that occurs in dim lighting is indicative of potential bloodstains.

Utilization of one or more presumptive tests can aid in blood identification. They can help the analyst's visual examination by highlighting potential stains, which are then collected for further testing. However, because these tests are presumptive they do not confirm the presence of blood. There are also several drawbacks to presumptive blood testing methods.

1.2.2 Limitations to Presumptive Tests

False positive results are important to consider when using catalytic color tests. This refers to the generation of a positive result in the absence of blood. This event occurs in multiple scenarios and is sometimes unavoidable. Exposure to items containing certain plant peroxidases will mimic a positive color change. Items that contain plant peroxidase include horseradish, potatoes, and cabbage (10, 11, 12, 18, 19).

Positive results will also occur with blood from animals. Chemical oxidants can cause false positives because they catalyze the reaction similar to heme, so even when heme is not present, there is a still a positive color change (11).

False negatives occur when blood is present, but a test is unable to detect it. These can occur in both presumptive and confirmatory tests when blood is too dilute or has become insoluble with age. Studies have also shown that false negatives can occur when blood has been in contact with certain household products due to the product's composition. Some chemicals, tannins, antioxidants, and vitamin C, have evidence of altering presumptive tests (20, 21, 22, 23).

1.2.3 Confirmatory Testing for Blood

Lateral flow chromatographic immunoassay cards confirm the presence of blood and avoid the false positives with peroxidases associated with presumptive tests. They are also very sensitive, quick, safe, and highly species specific. The cards contain 3 different sections: the sample well, test region, and control region. To begin the test, a sample extract is added to the sample well. The sample well holds mobile monoclonal antibodies that are bound to a dye. Since the sample well contains anti-human hemoglobin or anti-human glycophorin A monoclonal antibodies, the confirmatory tests are specific to human/primate blood. Monoclonal antibodies specifically react to a single epitope on its complementary antigen. If the target antigen is detected in the extract (glycophorin A or hemoglobin) the antibody will bind to the antigen and form an antigen-antibody complex (11, 23, 24). The test region contains immobilized polyclonal antibodies that trap the antibody-antigen complex as it travels up the card's membrane. The control region consists of an antibody dye conjugate, which appears to indicate the test is working properly regardless of the presence of blood. The antibodies in the control region are designed to bind to certain antibodies that have migrated from the sample area and act as a quality control measure for the confirmatory tests. If the control line is not present for a test, the result is considered inconclusive (11, 24, 25).

1.2.4 Rapid Stain Identification Series (RSID™)

The RSID™-Blood test is reported to be very sensitive (25). Since this test identifies the presence of glycophorin A, a red blood cell membrane protein, it is specific for blood and does not cross react with other body fluids (26). Glycophorin A tends to result in fewer false negatives when exposed to detergents containing active oxygen (found in many cleaners) (27). The use of multiple confirmatory tests is useful because they target different components of blood. Comparison of results between the two types of confirmatory tests can help to identify possible false negatives.

1.2.5 HemaTrace®

HemaTrace® targets human hemoglobin rather than glycophorin A. Some studies support the use of HemaTrace® over RSID™-Blood. These studies claim that HemaTrace® is more sensitive and exhibits better results for faint stains (22, 28). One study also reported that this test performed better on latent stains that first received visual enhancements, such as luminol. The limit of detection for HemaTrace® was determined to be around 0.05-0.07 µg hemoglobin/mL (22, 28). One study found that positive results were unaffected by multiple interferents (22).

1.2.6 Limitations to Confirmatory Tests

False negative results have been known to occur due to the high dose hook effect. The high dose hook effect occurs when there is too much antigen, which can happen when too much blood is added to the sample well. When there is too much hemoglobin, the excess hemoglobin does not allow the antibody and antigen complex to bind in the sample region. Instead, the excess hemoglobin moves toward the test region without the dye (23). Although there is blood present, there is no line at the test region, resulting in a false negative. There is also a chance of cross reactivity with upper primates and ferrets when using HemaTrace® (28). In most cases, the ability to differentiate between human, primate and ferret blood is not necessary, but it is important to note that it is not always guaranteed to be confirmatory for human blood.

1.3 Purpose of Study

The 2021 study conducted in this laboratory included a wide range of interferents, ranging from lab chemicals to household products. The products were chosen specifically because they have been noted in previous research to generate false negative results. In that study, bloodstains exposed to chemical ingredients found in common household products, as well as to the products themselves, were tested over a period of 106 days (22). The purpose of the current study was to test the same treated samples and compare the presumptive and confirmatory results after an extended period of time of approximately three years. This experiment sought to provide insight that could be useful in casework, especially for potential blood evidence that has been exposed to similar chemical interferents over a long period of time.

2. MATERIALS AND METHODS

2.1 Sample Preparation

Based upon the literature, an inclusive selection of chemicals, wines, juices, teas and coffee, cleaners, and leather substrates were chosen. All the samples used in this experiment were prepared in 2021 by a previous researcher in this laboratory (Table 1). The chosen interferents below were made in solution with either water or phosphate-buffered saline (Fisher Scientific, Fair Lawn, New Jersey). Ascorbic acid (Fisher Scientific, Fair Lawn, New Jersey), chlorogenic acid (Fisher Scientific, Fair Lawn, New Jersey), hydrogen peroxide (Fisher Scientific, Fair Lawn, New Jersey), oxalic acid (Fisher Scientific, Fair Lawn, New Jersey), quebracho extract (YarnTreeUSA™, Etsy store, Roanoke, Virginia), chestnut extract (YarnTreeUSA™, Etsy store, Roanoke, Virginia), sodium percarbonate (ACROS Organics™), catechin (Cayman Chemical, Ann Arbor, Michigan), proanthocyanidins (BulkSupplements.com®, Henderson, Nevada), and theaflavin (Life Extension, Fort Lauderdale, Florida) were the interferents used (22).

Table 1. Procedure for Preparing Chemical Interferents

Interferent	Preparation Method
1% ascorbic acid	0.25 g of ascorbic acid + 25 mL of deionized water and mixed until clear solution formed.
10% ascorbic acid	2.5 g of ascorbic acid + 25 mL of deionized water and mixed until clear solution formed.
10% chlorogenic acid	2.5 g of chlorogenic acid crystals + 25 mL of buffer and mixed until dissolved and an opaque solution formed.
1% catechin	0.25 g of +(-) catechin hydrate crystals + 25 mL of buffer and mixed until a tan solution formed.
10% catechin	2.5 g of +(-) catechin hydrate crystals + 25 mL of buffer and mixed until a tan solution formed.
10% hydrogen peroxide	8.3 mL of 30% hydrogenic peroxide + 16.7 mL of deionized water and mixed to form a uniform solution.
15% sodium percarbonate	3.75 g of sodium percarbonate + 25 mL of deionized water and mixed until a clear solution formed.
10% oxalic acid	2.5 g of oxalic acid 25 mL of deionized water and mixed until a clear solution formed.
5% proanthocyanidins	1.125 g of grape seed extract + 25 mL of deionized water and mixed until a dark brown solution formed.
2% quebracho extract	0.5 g of quebracho natural dye + 25 mL of deionized water and mixed until a dark brown solution formed.
20% quebracho extract	5 g of quebracho natural dye + 25 mL of deionized water and mixed until a brown solution formed.
2% chestnut extract	0.5 g chestnut natural dye + 25 mL of deionized water and mixed until a brown solution formed.
20% chestnut extract	5 g chestnut natural dye + 25 mL of deionized water and mixed until a brown solution formed.
2% theaflavin	2 capsules of theaflavin standardized extracts + 25 mL of deionized water and mixed until a dark brown solution formed.
20% theaflavin	26 capsules of theaflavin standardized extracts + 25 mL of deionized water and mixed until a dark brown solution formed.

2.1.1 Blood and Dilution Preparation

This experiment utilized bloodstains remaining from the original experiment. Blood was collected from two anonymous volunteers in compliance with the Institutional Review Board (IRB) of Boston University School of Medicine (Boston, Massachusetts, USA). The blood was collected using a BD Vacutainer® without preservatives and was stored frozen at the time of the original experiment (22).

A serial dilution procedure was used to dilute the donor's blood into 1:10, 1:50, 1:100, 1:500, and 1:1000 ratios. After the liquid blood was added to the deionized water, the solution was mixed using a Vortex Mixer (Fisher Scientific, Fair Lawn, New Jersey). Beginning with the 1:10, 100 µL of blood was added to 900 µL of deionized water. The 1:50 dilution was prepared by adding 20 µL of blood to 980 µL of deionized water. To prepare the 1:100 dilution, 100 µL of the previously made 1:10 dilution was added to 900 µL of deionized water. To prepare the 1:500 dilution, 100 µL from the 1:50 solution was added to 900 µL of deionized water and mixed. Lastly, to prepare the 1:1000 dilution the same step was repeated using the 1:100 dilution. From these dilutions, 2 µL of blood was added directly on the designated cotton swatches or leather substrates. Undiluted liquid blood was used to prepare neat stains in the same manner. When the cleaner interferents were created, 2 µL of blood was added before the cleaner to simulate a bloodstain that had been cleaned up . For the chemical interferents, Donor 1 blood was used. For the household products, Donor 2 blood was used. The cotton swatches used to deposit blood and chemical interferents were 4x4 gridded Texwipe™ All-Purpose Cotton Wipers (Fisher Scientific, Fair Lawn, New Jersey) (22).

2.1.2 Household Products and Cleaners

Various kinds of commonly found genuine leather products were purchased. They included a black leather jacket (Chadwicks®, United States of America), a red handbag (Wilson’s leather, India), brown Timberland® boots (Timberland LLC, Stratham, New Hampshire), and 2 wallets, light brown and red-brown (Tommy Hilfiger, United States of America) (22).

A variety of household products and cleaners were included because of the different chemical compositions and production methods. Five milliliters of each product were used to soak the substrates, which were then dried overnight. These samples were then stored at room temperature in designated folders. Ground coffee (Foldgers Classic Roast®, New Orleans, Los Angeles), Earl Grey black tea (Twinings®, London, United Kingdom), and green tea (Good & Gather Organic, Minneapolis, Minnesota) were prepared as directed on the packages. The lime, lemon, and orange juices were squeezed directly from the fruit to preserve the integrity of the potential interferent (22). The three types of wines in this study contained high amounts of tannin, a known interferent for forensic blood tests. The wines selected were 2018 Cabernet Sauvignon (John Cellars, Hopland, California), 2017 Langhe Nebbiolo (Azienda Agricola COS, Moorestown, New Jersey), and 2015 Rioja (Marques de Caceres, Cenicero, Spain) (22).

Lastly, OxiClean™ (Church & Dwight Co., Ewing, New Jersey), Nature Choices Oxy-Boost (Pacific Sand Inc., Kenosha, Wisconsin), Lysol containing hydrogen peroxide (Lysol®, Parsippany, New Jersey), and Bar Keeper’s Friend® (BKF) (SerVause Laboratories, Inc., Indianapolis, Indiana) were the selected cleaners due to their active ingredients. Lysol and OxiClean™ were sprayed directly onto the cotton and the Oxy-Boost and BKF were prepared using 7.6 g of cleaner into 500 mL of deionized water. One set of these soaked swatches were used unwashed (dried overnight), while the other was handled in a manner to mimic a washing effect. This involved adding the cleanser-soaked swatches into water for 5 minutes, and then drying them overnight (22).

2.1.3 Positive and Negative Controls

Positive controls for all tests were prepared by using six blood dilutions. Positive controls of neat bloodstains were prepared by depositing 2 μL of undiluted liquid blood from Donor 1. The same steps were repeated using 1:10, 1:50, 1:100, 1:500, and 1:1000 dilution liquid blood solutions from Donor 2 (22). The cotton swatches were left to dry at room temperature overnight; no chemical interferents were added. The negative control was an untreated cotton swatch, containing no blood or interferent. All controls were stored separately in designated folders at room temperature.

2.2 Preparing Presumptive Test Reagents

Preparation of these reagents aligned with the lab's standard operating procedures. Specific working volume conditions, storage preferences, and cutoff times were kept the same throughout the 2021 study and this current study. Both reagents were made prior to testing the substrates.

2.2.1 Kastle-Meyers Preparation

The KM stock solution was prepared by adding 2 g of phenolphthalein and 20 g of sodium hydroxide (Fisher Scientific, Fair Lawn, New Jersey) into a round bottom flask containing 100 mL of deionized water and a layer of zinc granules (Fisher Scientific, Fair Lawn, New Jersey). The solution was mixed until colorless. After the stock solution was made, the working solution was prepared. This was created by diluting the previously refluxed solution with methanol in a 1:5 ratio. Both the stock and working solutions were kept refrigerated in an amber bottle until testing (22).

2.2.2 Orthotolidine Preparation

The O-TOL solution was prepared using 4.5 g of orthotolidine (Fisher Scientific, Fair Lawn, New Jersey) dissolved in a solution of 120 mL ethanol (Fisher Scientific), 90 mL glacial acetic acid (Fisher Scientific, Fair Lawn, New Jersey), and 90 mL of deionized water. The solution was stored in an amber bottle and refrigerated until use (22).

2.2.3 Sample Controls

Prior to sample testing, positive and negative controls were tested to ensure reagents were working properly and were not contaminated. There were multiple negative control substrates including untreated cotton swatches, untreated cotton swabs (for leather) and cotton swatches treated with each specific interferent with no blood. Negative controls worked properly if results remained negative (no characteristic color change) after 15 seconds.

2.3 Presumptive Test Procedure

Before any data was collected, a picture was taken of each of the substrates containing dilutions to establish the blood visibility after the 3-year period. A four-legged camera stabilizer was used in order to create consistent lighting conditions, angle, and distance conditions for each of the photos.

Each blood dilution exposed to the household products, cleaning supplies, leather and chemical interferents was tested utilizing both presumptive tests. Testing with KM and O-TOL was performed in duplicate due to the limited sample quantities. The presumptive testing was completed by directly adding the reagent on a stain cut from the gridded substrates, which were placed on a labelled spot plate. For the leather substrates, a swab transfer method was used due to the difficult nature of cutting the thick substrate. This method involved using a sterile cotton swab to swab the leather. The appropriate reagents were added directly onto the swabs. To begin the procedure, a drop of either reagent was added to the cutting or swabs, then a drop of hydrogen peroxide (the oxidizer) was applied. The final result of the test was recorded immediately after 15 seconds. The presumptive testing procedure was the same for both KM and O-TOL, regardless of collection method.

2.4 Confirmatory Test Procedure

Confirmatory testing was performed directly after the presumptive testing using two common brands of immunoassay cards, RSID™-Blood (Independent Forensics, Lombard, Illinois) and HemaTrace® (Abacus Diagnostics®, West Hills, California). Only the neat stains with household products were used for confirmatory testing. Although these substrates had previously been tested in triplicate, due to limited sample, the confirmatory testing for the 3-year time point was performed in duplicate.

2.4.1 RSID™ Blood Procedure

A cutting of the 1:500 dilution was added to a tube filled with 500 µL of the RSID™ buffer. This buffer was included with the immunoassay card. During the 30-minute extraction step, the samples were placed on a Thermolyne 24 Rotomix 50800 Orbital Shaker (Fisher Scientific, Fair Lawn, New Jersey) and mixed. Once the extraction was completed, the samples were vortexed and 100 µL of the liquid extract was placed in the sample well of the card. The results were recorded once it turned positive, or after 10 minutes. If there was not a line on the test region after 10 minutes, it was considered negative.

2.4.2 HemaTrace® Procedure

A cutting of the 1:500 dilution was added to a tube filled with 500 µL of the HemaTrace® buffer. The samples were extracted for 30 minutes on a Thermolyne 24 Rotomix 50800 Orbital Shaker (Fisher Scientific, Fair Lawn, New Jersey) and vortexed before testing. When HemaTrace® cards were used, 80 µL of the sample extract was placed on the sample well, and the results were recorded for up to 10 minutes. The sample volume differed from the original study (200 µL) due to a change in procedure by Abacus Diagnostics®.

2.4.3 Interpreting Immunoassay Cards

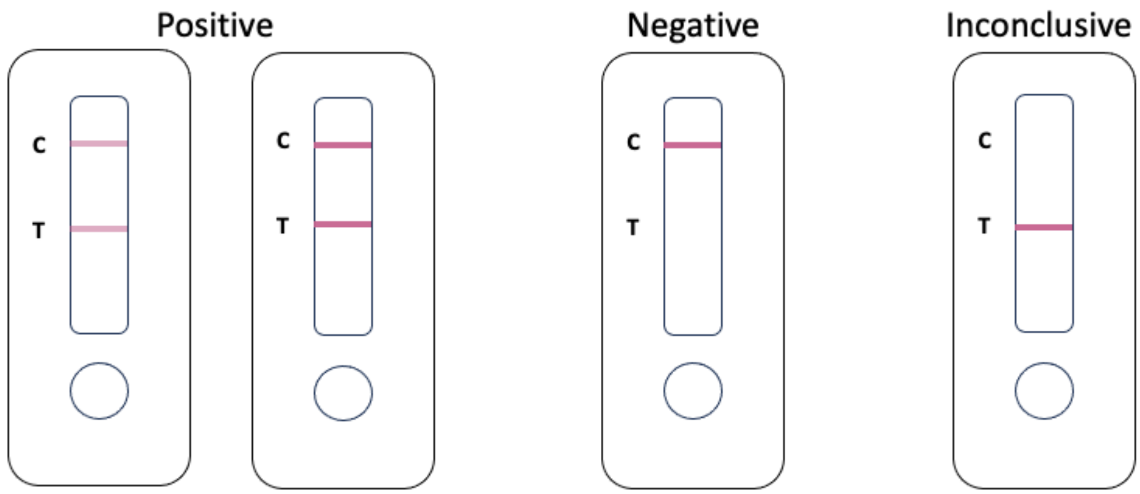

Figure 1. Possible Results for Confirmatory Blood Testing
This represents a visual of possible results that are used to interpret the confirmatory blood testing in this study. These results are represented by both brands, RSID™ and HemaTrace®.

Positive results have two colored bands, one at the control line and one at the test line (Figure 1). These bands varied in darkness but even faint lines were considered positive. The middle image indicates a negative result, where only the control line is visible. The last image on the right represents an inconclusive result. This result occurs when only one line on the test region was present on the card.

3. RESULTS

3.1 Preliminary Visual Inspection of Stains

Since all blood testing began with a visual inspection, each stain was carefully inspected for unique features and indication of where the blood had been previously deposited. If a substrate did not have the significant red or red-brown color, this could have been overlooked as a piece of evidence. In this study, presumptive testing still occurred regardless of stain visibility, however each substrate's stain color was recorded. If there was red or red-brown coloring, but the interferent changed the color of the substrate, this could have masked the presence of blood.

3.1.1 Positive and Negative Controls

A stain was visible for neat and 1:10 dilution stains. The 1:50 dilution stain was very difficult to visualize and was only discernable using the macro setting on the camera. For the remaining dilutions, 1:100, 1:500 and 1:1000, no staining was observed. The negative control was the white cotton swatch and white tip swabs, where no visual stains were observed.

3.1.2 Chemical Interferents

Both concentrations of the ascorbic acid interferent caused the white substrate to turn an overall yellow color. The ascorbic acid neat bloodstain and the 1:10 dilution exhibited red-brown staining, but the remaining dilutions did not. The other dilutions were noted to have a distinct ring around the deposited stain, but the inner center of the stain was white, creating a contrast from the yellowing of the acid.

The chlorogenic acid caused the development of a light yellow on the negative control substrate. The neat stain was visible and the 1:10 dilution was faint. The remaining bloodstain dilutions were not visible.

The negative substrate control for both catechin concentrations was light orange in color, but darker for the 10%. The 10% catechin exhibited a darker overall color, and there was some transfer of color onto the package that the substrates were stored in. Red-brown staining was observed for neat bloodstain and the 1:10 dilution. For 1% catechin, very faint red-brown staining was observed for 1:50 and 1:100 dilutions. There was no visible staining on the remaining dilutions. For 10% catechin, there were only visible stains on neat, 1:10 dilution, and 1:50 dilution.

The hydrogen peroxide samples retained the substrate's original white color. The neat blood was visible, but the typical red-brown color appeared muted. The 1:10 dilution was very faint and difficult to visualize. The remaining blood stain dilutions were not visible.

Similarly to hydrogen peroxide, sodium percarbonate retained the substrate's white color. The neat blood appeared a muted red-brown. The 1:10 dilution was very difficult to visualize and 1:50 and 1:100 dilutions could only be discerned by using the macro setting on the camera. The remaining 1:500 and 1:1000 dilution bloodstains were not visible.

Oxalic acid remained white on the substrate and neat blood was distinctly visible. However, the 1:10 dilution appeared to have faded and turned a light orange. The remaining dilutions had hints of the faded orange color, but there were no distinct stain shapes. Another observation was that the manila envelope that contained the treated samples was very brittle and breaking into pieces. None of the other chemical interferents exhibited this same effect. This was likely due to the strength of the acid and may be indicative of potential degradation of evidence.

The proanthocyanidins turned the substrate a copper color. The neat blood was apparent. The 1:10 dilution was difficult to distinguish between the copper substrate and the red-brown stains, but this could have been easily overlooked without prior knowledge of its existence. The remaining dilutions were not visible.

Both concentration of quebracho extract turned the substrate brown, with the 20% being a much darker brown, and both 2% and 20% quebracho extract exhibited visible neat stains. The remaining dilutions for the two concentrations were not visible.

The 2% chestnut extract turned the substrate an overall beige color, while the 20% was considerably darker brown. The neat bloodstain and 1:10 dilution stains for both concentrations were visible. The 1:50, 1:100, 1:500, 1:1000 dilution stains did not exhibit the distinct red-brown color that is normally associated with blood. A lighter beige color outlined the stains that allowed for their visualization.

Although both concentrations of theaflavin turned the substrate brown, the neat bloodstains remained clear and visible with a distinct red-brown color. The 1:10 and 1:50 dilution stains were faint but still visible. The 20% theaflavin was much darker and the deep brown color of the substrate was uneven making some sections darker than others. If a bloodstain was deposited on these sections with the darker theaflavin, it would be much harder to distinguish.

3.1.3 Household Products

For all three wines, the substrate turned a light pink color. The only bloodstains that exhibited any noticeable color were the neat and 1:10 dilution. The remaining dilutions were not visible. The colors of both the dilute blood and wine looked similar, which would explain why the bloodstains were unable to be differentiated from the wine.

The lemon and lime juices turned the substrate a beige color. Orange juice turned the substrate a slight yellow color. The neat blood was visible and 1:10 dilution looked faint but still exhibited some red-brown color. The remaining dilutions did not develop red-brown color, but the stains exhibited a lighter contrast from the background, with a distinct border.

The coffee, black tea and green tea all revealed a slight variation in the substrate color. Black tea turned light yellow, green tea was light orange, and coffee was beige. All three neat stains were visible and dark red-brown. The 1:10 dilution for the three products was slightly visible with a light red-brown color. The remaining dilutions exhibited no color but contained a darker outer ring which aided in the differentiation between the substrate color and the stain. Without the red-brown color, visual inspection was difficult.

3.1.4 Cleaners

Both the unwashed and washed Lysol kept the substrate's original white color. The neat blood appeared a typical red-brown for both washed and unwashed samples. The 1:10 dilution was a faint orange, and the remaining dilutions were not visible.

Both unwashed and washed Oxi-Clean™ had white substrates except for on neat blood. The neat bloodstains were not a characteristic color for blood. The entire neat substrate looked yellow, making it difficult to visualize. The other dilutions were not visible.

Both unwashed and washed Oxy-Boost substrates remained white. As observed with the Oxi-Clean™, neat blood was not a characteristic color. It was dark brown and had a smudged appearance. The neat blood's smudged appearance made it not clear where the original deposit of blood was, which was not observed in any other interferents except BKF. Unwashed and washed 1:10 dilutions were extremely faint orange. The remaining dilutions were not visible.

The neat staining on washed and unwashed BKF showed a light red-brown color. The only difference was that unwashed neat bloodstains retained a uniform circular shape, while the washed bloodstains looked smudged. The 1:10 dilution was faint for both the washed and unwashed, and the rest of the dilutions were not visible.

3.1.5 Leather Substrates

Visual inspection of the leather substrates was much more difficult than the cotton swatches. The only bloodstains visible with the black leather jacket were neat and 1:10 dilution, but visualization was not based on the coloring. These stains were only slightly visible due to the thick, shiny consistency of the blood that was able to seen in oblique lighting. Stains beyond 1:10 were not visible on the jacket.

For the leather boot substrate, neat blood was dark and apparent as the stains dried into a thick droplet. Stains of 1:10, 1:50, 1:100 and 1:500 diluted blood on the boots were able to be observed due to textured outline left by the dried bloodstain, with a slight difference in color. 1:1000 stain was not distinguishable.

All dilutions were easily noticed for the light brown leather wallet. As expected, as blood became more dilute, the stains were harder to see. However, it was clear to examine the exact size of the blood droplets on the wallet. As for the red-brown leather wallet, neat blood deposited on was the only distinguishable bloodstain. The remaining dilutions were unable to be visualized on the red-brown wallet.

Lastly, the red leather handbag had staining observed for neat, and 1:10 blood dilution. The remaining dilutions were unable to be distinguished against the red leather substrate similar to the red-brown wallet.

3.2 Positive and Negative Controls for Presumptive Testing

No characteristic color change was produced while testing the negative controls using KM and O-TOL reagents, resulting in the expected negative results (Table 2). The negative results were consistent for every substrate with chemical treatment, as well as the original cotton swatch that had no interferents. Testing of positive controls of diluted bloodstains using KM reagent since the 106-day mark in the 2021 study was negative. This precluded further testing with the chemical interferents using KM on treated diluted blood samples. Further KM testing was conducted only with neat bloodstains as they yielded the expected positive result. Although the testing was performed around three years and four months after sample preparation, it is unusual for KM to not exhibit positive results due to its sensitivity. Since the negative results were as expected, the substrate and reagent contamination was ruled out as a contributor. O-TOL results for all positive controls, and the negative control were acceptable.

Table 2. Positive and Negative Control Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result; no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer	Orthotolidine
Neat	+	+
1:10	-	+
1:50	-	+
1:100	-	+
1:500	-	+
1:1000	-	+
Negative Control	-	-

3.3 Results for Presumptive Testing with Chemical Interferents

3.3.1 Ascorbic Acid

For the more concentrated 10% ascorbic acid, the KM test did not work for the neat stain tested in 2021 (Table 3). However, the test was positive in 2024 and resulted in bright and quick results. The atypical brown color that developed with O-TOL was consistent between the neat bloodstain from both years. The 1% ascorbic acid produced one additional positive (1:50) in 2024 than in 2021 for O-TOL (Table 4).

Table 3. 10% Ascorbic Acid Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result; no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	-	+	-	-
1:10	**n/a**	**n/a**	-	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 4. 1% Ascorbic Acid Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result; no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	-	+
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.3.2 Chlorogenic Acid

Chlorogenic acid results were consistent between both years for both KM and O-TOL (Table 5). The only difference between 2021 and 2024 was that the neat, 1:10, 1:50, and 1:100 dilutions were initially positive. However, within the 15 second window the positive blue turned into a negative, atypical purple.

Table 5. 10% Chlorogenic Acid Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed. '+-' signifies an original positive color change of blue, that eventually turned into an abnormal color. For this result, the final color that was observed at 15 seconds is the color of the cell.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	-	-	+-	-
1:10	**n/a**	**n/a**	+-	-
1:50	**n/a**	**n/a**	+-	-
1:100	**n/a**	**n/a**	+-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.3.3 Catechin

The 10% catechin results were identical between the years with both reagents. Neat blood with KM for 2021 and 2024 was negative and turned an atypical dark red (Table 6). The 1% catechin in 2021 had a positive neat KM result, but 2024 had an atypical dark red result (Table 7). Similar to 10% catechin, the 1% catechin in 2024 produced negative, atypical purple results for the neat, and 1:10, 1:50, and 1:100 dilutions.

Table 6. 10% Catechin Results Compared to 2021 Results

Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result is different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	-	-	-	-
1:10	**n/a**	**n/a**	-	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 7. 1% Catechin Results Compared to 2021 Results

Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed. '+-' signifies an original positive color change of blue, that eventually turned into an abnormal color. For this result, the final color that was observed at 15 seconds is the color of the cell.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	-	+-	-
1:10	**n/a**	**n/a**	+-	-
1:50	**n/a**	**n/a**	+-	-
1:100	**n/a**	**n/a**	+-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.3.4 Hydrogen Peroxide

Table 8 outlines the identical results observed between both years for both reagents. The stains exhibited positive results for neat blood using KM, and neat through 1:100 dilutions for O-TOL.

Table 8. 10% Hydrogen Peroxide Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result; no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.3.5 Sodium Percarbonate

The neat and diluted blood exposed to sodium percarbonate exhibited an inconclusive result with O-TOL in 2021. An inconclusive result was recorded because the color change occurred before the oxidizer was added. In 2024, O-TOL was positive for all bloodstains. There were no changes observed for neat blood tested with KM between the years (Table 9).

Table 9. 15% Sodium Percarbonate Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. '*' indicates an inconclusive result, which in this case, occurred when the stain had a color change before the presence of the oxidizer.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	*	+
1:10	**n/a**	**n/a**	*	+
1:50	**n/a**	**n/a**	*	+
1:100	**n/a**	**n/a**	*	+
1:500	**n/a**	**n/a**	*	+
1:1000	**n/a**	**n/a**	*	+
Negative Control	-	-	-	-

3.3.6 Oxalic Acid

Both neat and 1:10 stains tested with O-TOL in 2021 and 2024 exhibited atypical colors. This color was yellow-green and was consistent for both stains. No changes were observed for neat blood tested in KM between the years (Table 10).

Table 10. 10% Oxalic Acid Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-'refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	-	-
1:10	**n/a**	**n/a**	-	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.3.7 Proanthocyanidins

Testing in both 2021 and 2024 produced negative results for KM. The negative results also generated an atypical maroon color. In 2024, O-TOL was negative and atypical dark brown color was observed for neat and the 1:10 dilution (Table 11).

Table 11. 5% Proanthocyanidins Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result is different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	-	-	-	-
1:10	**n/a**	**n/a**	-	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.3.8 Quebracho Extract

KM produced an atypical result that was an off-white beige color in 2024 (Table 12). In 2024, there were no O-TOL positives, and the neat bloodstain produced an atypical dark brown/black color. The remaining dilutions for 20% quebracho were inconclusive in 2024. Results were challenging to interpret because the stain's color was masked by the addition of the reagent, causing the entire cutting to turn black.

Using O-TOL with 2% quebracho extract caused atypical colors for neat, 1:10, 1:50, and 1:100 bloodstains in both years. These colors, however, were different (Table 13). In 2021, O-TOL caused the stains to become green. In 2024, the stains became a dark brown/black color.

Table 12. 20% Quebracho Extract Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed. '*' indicates an inconclusive result, which in this case, occurred when the stain was masked by the addition of the reagent causing the entire cutting to be colored black.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	-	+	-
1:10	**n/a**	**n/a**	-	*
1:50	**n/a**	**n/a**	-	*
1:100	**n/a**	**n/a**	-	*
1:500	**n/a**	**n/a**	-	*
1:1000	**n/a**	**n/a**	-	*
Negative Control	-	-	-	-

Table 13. 2% Quebracho Extract Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle--Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	-	-
1:10	**n/a**	**n/a**	-	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.3.9 Chestnut Extract

In 2024, neat and 1:10 dilution bloodstains with 20% chestnut extract had a negative and atypical brown/black result with O-TOL; the neat bloodstain was negative with KM (Table 14). For 2% chestnut extract, using KM on the neat stain in 2021 resulted in a change from positive to a light orange, negative result (Table 15). In 2024, stains from neat to 1:100 dilutions exhibited an atypical dark brown/black color. (Table 15).

Table 14. 20% Chestnut Extract Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	-	+	-
1:10	**n/a**	**n/a**	-	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 15. 2% Chestnut Extract Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed. '+-' signifies an original positive color change of blue, that eventually turned into an abnormal color. For this result, the final color that was observed at 15 seconds is the color of the cell.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+-	+	+	-
1:10	**n/a**	**n/a**	-	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.3.10 Theaflavin

Negative and atypical beige KM results were consistent between 2021 and 2024 for neat blood on 20% theaflavin. For 20% theaflavin, O-TOL in 2021 resulted in atypical green color for neat and 1:10 dilution. In 2024, there was an atypical dark brown/black color for the neat stain and the remaining dilutions were inconclusive (Table 16). This similar result was seen with 20% quebracho extract (Table 12). This result meant that it was impossible to interpret any color changes due to the stain's color being masked by the addition of the reagent, which caused the entire cutting to turn black.

With 2% theaflavin, atypical results were seen using O-TOL for the neat, 1:10, 1:50, and 1:100 dilutions (Table 17). The color of these atypical results was different between the two time periods. In 2021, the color was green and in 2024 the color was dark brown/black. No change were observed for neat blood tested with KM between the years.

Table 16. 20% Theaflavin Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color correspond to the atypical color that was observed. '*' indicates an inconclusive result, which in this case, occurred when the stain was masked by the addition of the reagent causing the entire cutting to be colored black.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	**-**	**-**	**-**	**-**
1:10	**n/a**	**n/a**	**-**	*
1:50	**n/a**	**n/a**	**-**	*
1:100	**n/a**	**n/a**	**-**	*
1:500	**n/a**	**n/a**	**-**	*
1:1000	**n/a**	**n/a**	**-**	*
Negative Control	**-**	**-**	**-**	**-**

Table 17. 2% Theaflavin Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	-	-
1:10	**n/a**	**n/a**	-	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.4 Results for Presumptive Testing with Household Products

3.4.1 Wines

In 2021, when O-TOL was used on 1:50 and 1:100 dilutions, the color changed from positive to purple within 15 seconds for cabernet sauvignon samples. In 2024, neat bloodstain through 1:100 dilution all produced positive results (Table 18). With Langhe Nebbiolo in 2021, O-TOL results were positive for neat and 1:10, but turned from positive to purple for the 1:50 and 1:100 dilutions, which was also seen with cabernet (Table 19). In 2024 however, both Langhe Nebbiolo and cabernet had turned positive for 1:50 and 1:100. Langhe Nebbiolo even lost a false negative and gained a positive result for 1:500 in 2024. With Rioja, stains tested in both years exhibited positive results for neat blood using KM, and neat through 1:100 dilutions for O-TOL (Table 20).

Table 18. Cabernet Sauvignon Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative, but the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+-	+
1:100	**n/a**	**n/a**	+-	+
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 19. Langhe Nebbiolo Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative, but the cell color corresponds to the atypical color that was observed.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+-	+
1:100	**n/a**	**n/a**	+-	+
1:500	**n/a**	**n/a**	-	+
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 20. Rioja Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	**-**	**-**
1:1000	**n/a**	**n/a**	**-**	**-**
Negative Control	**-**	**-**	**-**	**-**

3.4.2 Tea and Coffee

Tables 21, 22, and 23 outline the results for the teas and coffee. Black tea produced one additional false negative in 2024 (1:500 dilution) than in 2021 (Table 21). Green tea also had an additional false negative (1:500 dilutions) than in 2021 (Table 22). Lastly, coffee (Table 23) had the same results between both years, with positive results from neat blood to 1:100 dilutions. No changes were observed for neat blood tested with KM between the years and across the teas and coffee tested.

Table 21. Black Tea Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	+	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 22. Green Tea Results Compared to 2021 Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 23. Coffee Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.4.3 Citrus Juices

Tables 24, 25, and 26 outline the results of the citrus juices. Between both years, lemon juice results stayed consistent for O-TOL and KM (Table 24). With lime juice, KM had a negative result for neat blood in 2024. O-TOL results remained the same for both years (Table 25). Orange juice had one additional negative result in 2024 for the 1:500 dilution (Table 26).

Table 24. Lemon Juice Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 25. Lime Juice Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	-	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 26. Orange Juice Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	+	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.4.4 Cleaners

Washed Lysol tested with O-TOL in 2021 produced an atypical green result that turned blue within the 15 second window for the neat stain. This did not occur in 2024 (Table 28). Results remained the same for the unwashed Lysol samples between both reagents. Similarly, to washed Lysol, the 2021 neat bloodstain on unwashed OxiClean® had an atypical green result that turned blue within the 15 second window while the 2024 did not. Unwashed OxiClean® did not have a positive KM result in 2024, as well an additional false negative for the 1:10 dilution (Table 29). Results remained the same for both reagents for washed OxiClean® (Table 30). In 2024, the neat stain with the washed and unwashed Oxy-Boost produced an inconclusive result (Tables 31, 32). This occurred when the stain had a color change of bright red before the oxidizer was added. With dilutions 1:100 through 1:1000 however, an atypical green color appeared for both the washed and unwashed Oxy-Boost. For neat stains with O-TOL, an atypical green changed to blue (positive) in 2021 for washed BKF (Table 34). Unwashed BFK produced similar results for both reagents between the years (Table 33).

Table 27. Unwashed Lysol Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	**-**	**-**	+	+
1:10	**n/a**	**n/a**	**-**	**-**
1:50	**n/a**	**n/a**	**-**	**-**
1:100	**n/a**	**n/a**	**-**	**-**
1:500	**n/a**	**n/a**	**-**	**-**
1:1000	**n/a**	**n/a**	**-**	**-**
Negative Control	**-**	**-**	**-**	**-**

Table 28. Washed Lysol Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell, unless result was different. '-+' represents an atypical color, in this case green, that changed into a positive blue color within the required 15 seconds.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	-+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 29. Unwashed OxiClean® Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. '-+' represents an atypical color, in this case green, that changed into a positive blue color within the required 15 seconds.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	-	-+	+
1:10	**n/a**	**n/a**	+	-
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 30. Washed OxiClean® Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	-	-
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 31. Unwashed Oxy-Boost Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed. '*' indicates an inconclusive result, which in this case, occurred when the stain had a color change before the presence of the oxidizer. This premature color change is represented by the cell color.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	*
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 32. Washed Oxy-Boost Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. The atypical colors are considered negative and the cell color corresponds to the atypical color that was observed. '*' indicates an inconclusive result, which in this case, occurred when the stain had a color change before the presence of the oxidizer. This premature color change is represented by the cell color.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	*
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 33. Unwashed BKF Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	-	-
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 34. Washed BKF Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different. '-+' represents an atypical color, in this case green, that changed into a positive blue color within the required 15 seconds.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	-+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	-
1:500	**n/a**	**n/a**	+	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

3.5 Results for Presumptive Testing on Leather Products

The results for leather products showed that the age of the stains did not affect the results for KM and O-TOL. All KM tests on neat stains, regardless of leather product, were positive. For the leather handbag, positive results were observed for the neat, 1:10, 1:50, and 1:100. 1:500 and 1:1000 dilutions were negative. This result was consistent in 2021 and 2024. For the leather jacket, there were no false negatives with O-TOL, regardless of the blood dilution or age. The leather boots gave positive O-TOL results for every blood dilution except for 1:1000 in 2024, however it was negative for both 1:500 and 1:1000 in 2021. Lastly, both the red-brown and light brown wallets had positive O-TOL results for all dilutions except 1:500 and 1:1000. This occurred in 2021 and 2024 (Tables 35, 36, 37, 38 and 39).

Table 35. Leather Handbag Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 36. Leather Jacket Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	+	+
1:1000	**n/a**	**n/a**	+	+
Negative Control	-	-	-	-

Table 37. Timberland® Boots Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell, unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	-	+
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 38. Red-Brown Wallet Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	-	-
1:1000	**n/a**	**n/a**	-	-
Negative Control	-	-	-	-

Table 39. Light Brown Wallet Results Compared to 2021 Results
Results with a '+' signify that a representative positive color change occurred. The '-' refers to a negative result, no color change. The colored cells correspond to the color observed for the reaction. Both duplicates are represented by one cell unless the result was different.

Blood Dilutions	Kastle-Meyer 2021	Kastle-Meyer 2024	Orthotolidine 2021	Orthotolidine 2024
Neat	+	+	+	+
1:10	**n/a**	**n/a**	+	+
1:50	**n/a**	**n/a**	+	+
1:100	**n/a**	**n/a**	+	+
1:500	**n/a**	**n/a**	**-**	**-**
1:1000	**n/a**	**n/a**	**-**	**-**
Negative Control	**-**	**-**	**-**	**-**

3.6 Confirmatory Testing Results for Household Products

Confirmatory testing was only performed on neat stains due to the positive control's negative result for the 1:500 dilution. Previously, the control results were successful for the 1:500 dilution. This was not the case 3 years later; therefore, it was decided that confirmatory testing would be performed on neat blood only. When the neat blood was tested using the control substrate, it produced a positive result only for HemaTrace®. RSID™ was negative for the neat blood positive controls, and therefore was not used for the treated samples (Table 40). In the 2021 study, only the unwashed substrates treated with cleaners were tested. This was to keep any inconsistencies with the washing step from skewing the results. Table 41 compares the results between the 2021 1:500 dilutions and the neat blood results in 2024.

The household products that were the most strongly positive within both studies were the cabernet wine and leather jacket. Anything that was considered a faint positive in 2021 but a strong positive in 2024 may be due to a less concentrated bloodstain (1:500 dilution) being tested in 2021. Similarly, coffee, Langhe Nebbiolo wine, and black tea changed from being negative to becoming strongly positive from the previous study (Table 41). This may also be due to difference in blood dilution used. Besides the diluted samples, cleaners tested with neat blood did not produce positive confirmatory results. It is important to note that only the cleaning products tested negative for neat blood. Neat blood is rarely ever on evidence.

Orange juice, red-brown wallet, and the Timberland® boots had originally been a strong positive for 1:500, but turned faint positive in 2024 for neat blood (Table 41). This result was also surprising as neat blood was expected to produce strong positive confirmatory test results.

Since confirmatory blood testing is the last step in blood identification, and is often a precursor to DNA testing, it is concerning that it would test negative. If a negative confirmatory result occurred in case work, it would not make sense to submit the evidence for DNA testing. This means that a valuable piece of DNA could be missed and overlooked due to the bloodstained evidence being contaminated with any of the tested cleaners.

Table 40. Confirmatory Testing Results of Controls
A '+' represents a positive result. A '-' represents a negative result. The cell color presents the color of the band that was observed on the test line after the required 10 minutes. No color, or a white cell color, represents a negative result because no bands would be present on the test line, only on the control line.

	RSID™ 1	**RSID™ 2**	**HemaTrace® 1**	**HemaTrace® 2**
Positive Control	-	-	+	+
Negative Control	-	-	-	-

Table 41. Confirmatory Testing Results of Household Products Comparison
A summary of the confirmatory results of all the household products tested. The first column shows the average result of the triplicates from 2021 for HemaTrace®. A '+' represents a positive result. A '-' represents a negative result. The cell color represents the color of the band that was observed on the test line after the required 10 minutes. No color, or a white cell color, represents a negative result.

Household Product	HemaTrace® Summary 2021	HemaTrace® 1 2024	HemaTrace® 2 2024
Cabernet Sauvignon Wine	+	+	+
Langhe Nebbiolo Wine	-	+	+
Rioja Wine	**Faint** +	+	+
Lime Juice	**Faint** +	+	+
Lemon Juice	**Faint** +	**Faint** +	**Faint** +
Orange Juice	+	**Faint** +	**Faint** +
Black Tea	-	**Faint** +	**Faint** +
Green Tea	**Faint** +	**Faint** +	**Faint** +
Coffee	-	+	+
Unwashed Lysol	-	-	-
Unwashed OxiClean™	-	-	-
Unwashed Oxy-Boost	-	-	-
Unwashed BKF	-	-	-
Leather Handbag	**Faint** +	+	+
Leather Jacket	+	+	+
Timberland® Boots	+	**Faint** +	**Faint** +
Red-Brown Wallet	+	**Faint** +	**Faint** +
Light Brown Wallet	**Faint** +	+	+

4. DISCUSSION AND CONCLUSIONS

4.1 Summary of Findings

A typical crime scene may have all types of colors, patterns, and fabrics with potential bloodstains. Some fabrics may be more absorbent, making the stains harder to see. In correlation to the visual results, if a stain appears faint on the white fabric, it has the potential to be even less visible in a real-world scenario. Since the bloodstained substrates in this research were not touched for over three years, any changes that occurred may be similar to those in real cases where evidence has been stored for future testing, particularly with cold cases.

Time did not affect the KM results for blood exposed to 1% ascorbic acid, 15% sodium percarbonate, 2% and 20% theaflavin, Cabernet Sauvignon and Langhe Nebbiolo wine, black and green tea, orange juice, washed OxiClean™, washed Oxy-Boost and unwashed Oxy-Boost over a three-year period. For 10% ascorbic acid and 2% chestnut extract, time did improve the results as it became positive over the three years using KM reagent. On the other hand, 1% catechin, 20% quebracho , 20% chestnut, lime juice and unwashed OxiClean™ produced a false negative result after three years. For the rest of the chemicals and interferents tested with KM reagent, no change in the initial result was observed. Since only neat blood was able to be tested with KM, it is difficult to draw further conclusions from the results.

For O-TOL, only 10% ascorbic acid and lime juice were not impacted over time. There were also interferents that had the same results for both KM and O-TOL results during both testing periods. These interferents were 10% chlorogenic acid, 10% catechin, 5% proanthocyanidins, 10% hydrogen peroxide, 10% oxalic acid, Rioja wine, lime juice, 2% theaflavin, lemon juice, coffee, washed and unwashed Lysol, washed OxiClean, 2% quebracho extract, unwashed BKF, and all the leather substrates besides the leather boots. It was expected that catechin and chlorogenic acid would have results like coffee, because catechin and chlorogenic acid are types of antioxidants found in caffeinated beverages. However, the products containing similar chemical interferents did not have the same results. This may be due to differences in manufacturing, and the percentage of each chemical interferent in the products.

The primary goal of this research was to determine how long-term exposure to chemical interferents affected the occurrence of false negatives when testing for blood. The household products that produced the most false negatives (colorless reaction) in 2024 were unwashed OxiClean® and unwashed Lysol. The chemical interferents that had the best results were 15% sodium percarbonate, 10% hydrogen peroxide, and 1% ascorbic acid. The remaining chemical interferents did not have any positive results. The only bloodstains that tested positive for these interferents were the neat stains. The only interferent that continued to be O-TOL positive for the 1:1000 dilution was for the leather jacket and 15% sodium percarbonate. These interferents did not affect the high sensitivity of O-TOL, even after over 3 years.

There were also some interferents that performed better with O-TOL and KM in 2024 than in 2021. This can signify that the potency of the interferent has decreased over time and is no longer interfering with the blood and/or assay. Langhe Nebbiolo wine had a slightly more sensitive result in 2024 for O-TOL, as well as 1% ascorbic acid, 15% sodium percarbonate, leather boots, and cabernet On the other hand, for 20% quebracho extract, 20% theaflavin, 2% and 20% chestnut extract, the results were negative for O-TOL that occurred starting with neat blood. This result could indicate that the potency of these interferents increased over the three years.

It can be inferred that for some interferents, an initial negative result does not always mean that it will be negative in the future. This was especially true for Langhe Nebbiolo because in 2021, the majority of HemaTrace® results were negative. For 2024, the HemaTrace® was strongly positive for both duplicate stains. It is possible that a key ingredient found in that specific wine had lost its potency in 2024. This conclusion is supported with previous research that found that antioxidants and tannin amounts vary between different brands during the production stages (28, 29). Black tea and coffee were also originally negative and turned positive in 2024. For products such as Langhe Nebbiolo, black tea, and coffee, it can be concluded that blood may still be detected years later, even after an initial false negative result. Any stains with positive confirmatory results that originally were faint and turned into intense bands in 2024 were unable to be compared directly due to the dilution differences.

Orange juice, red-brown wallet, and the leather boots changed from a strong positive to a faint positive in 2024. Since neat blood was tested in this study, it was interesting to find that these products diminished in potency with HemaTrace® compared to the 1:500 dilution used in 2021. The manufacturing method between the wallet and boots may be similar in regards to the tannin concentration. Tannin, the interferent, might be causing the false negative by interfering with the hemoglobin detection. For the orange juice, the interferents of vitamin C and antioxidant might be a stronger concentration than the other juices, therefore blood exposure to them is diminishing the detectability for HemaTrace®.

All the unwashed cleaning products produced negative results for both HemaTrace® duplicates, which was also observed in 2021. One of the main ingredients of these cleaners is sodium percarbonate and hydrogen peroxide. These ingredients have been reported to cause false negatives in both the presumptive and confirmatory stages of forensic blood testing (30, 31, 32, 33). However, there were no false negatives in 2024 for sodium percarbonate alone. The cleaners still had many false negatives for presumptive and confirmatory tests, so sodium percarbonate is not the sole contributor to false negative results. The difference in sample preparation might also be correlated to the many false negative results. Cleaners were added only after the addition of blood, unlike the other household products. Perhaps soaking the substrate containing the bloodstains resulted in the bloodstains to not stick to the substrates as well, therefore being less bound and able to engage with the reagents better. Since the cleaners had consistent negative confirmatory results, the bloodstains might have lost biological activity when the cleaners were sitting on top of the blood and caused the cutting to not extract as well as the others.

Previous research reported that RSID™-Blood was able to detect blood that had been in contact with cleaners and that HemaTrace® produced more false negatives than RSID™ (31, 33). This claim was not supported in this current study because RSID™

indicated a false negative result for the positive neat control, even with no interferents. This may be attributed to the quality of blood and is possibly donor specific.

There were some unpredictable results when the diluted positive controls were used for KM and RSID™. There may be a correlation between the blood donor and these noted results. In the previous donor comparison study from 2021, donor 2 did not yield enough DNA for interpretable results (22). This same donor was responsible for the diluted controls that did not work with KM, and also for the RSID™ controls in 2024. There might have been some level of degradation of this donor's blood, which caused KM and RSID™ to have limited sensitivity. It would have been helpful if the donor remained consistent among both variables to narrow down possible explanations regarding the false negatives on the positive controls. Another explanation for the unexpected negative result on the positive control sample is the close proximity of the control samples to those that contained a harsh chemical reagent. Perhaps the false negative result occurred because one of the interferents inhibited the positive reaction from occurring.

4.2 Limitations and Further Considerations

Besides the effects of age and interferents, there are a few other reasons that could explain the false negatives. One would be subjective color interpretation. Since the previous study was performed by a different analyst, it could be that one person's perspective of the atypical color versus a positive color were dissimilar. It is possible that a darker shade of blue was still considered positive, but a blue color that had a greener tone was considered negative.

More consistent results and less false negatives were observed with leather. This might have to do with the collection technique used. A swab was taken rather than a cutting to test for leather products. There have been studies showing false negatives for more concentrated blood on leather products, but in this study the least dilute false negative for a leather substrate was a 1:500 dilution (26, 27). Furthermore, swabbing of the leather caused transfer of the dye, which could have made the development of the color harder to visualize.

Knowing that bloodstains have been deposited may cause bias because the data collector might be looking for any sort of positive blue/pink result regardless of how faint. In a real-world example, an analyst can't always visualize stains well before they test for them and they do not know whether or not they contain blood.

If a substrate had less false negatives in 2021 than 2024, it was hard to determine whether it was due to degradation, chemical interferent, or both. The atypical colors were most likely the result of the reagent and chemical interferents reacting. If there were less false negatives in 2024 than 2021, (which occurred with Langhe Nebbiolo wine, ascorbic acid, and Timberland® boots), it might have been due to the interferent losing its potency.

Lastly, false negative results that were consistent between both years could signify that it was likely the interferent caused the false negatives irrespective of the duration of exposure. These tests are normally very sensitive, so the negative results for the neat blood indicate the interferent being responsible.

4.3 Conclusions

A significant finding from this study is that bloodstains, when in contact with certain chemicals, could yield false negative results when tested for the presence of blood. This is especially true when blood gets diluted beyond 1:100 or has extended contact with the chemical interferent. However, there is not a clear time cutoff and some of the chemicals tested were unaffected by this. Another significant finding was that the more concentrated chemicals had less consistent results. One reason for this may be that the strong chemicals resulted in very dark staining, making interpretation difficult. The catalytic color test reactions might not have been bright enough to be visualized as a positive result on the dark substrate.

Lastly, since there were many false negatives in presumptive testing, it highlights the possibility of investigators failing to detect or collect probative bloodstains. For example, blood dilutions exposed to Lysol tested negative during both time periods, (day 106 and year 3). Upon visual inspection, any dilution above a 1:10 dilution was nearly impossible to visualize. Thus, it is unlikely that blood evidence such as this would be submitted for DNA analysis. This demonstrates why continued research toward understanding the causes of false negative results is important for the forensic community.

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